

## WHAT IS CLAIMED IS:

1. A method of analyzing a polynucleotide sample for one or more target sequences, comprising the steps of:

contacting a polynucleotide sample suspected of comprising one or more target sequences with: (i) a first signal probe which is capable of hybridizing to at least a portion of a first target sequence and producing a first detectable signal when hybridized thereto; (ii) a first quencher probe which is capable of hybridizing in quenching proximity to the first signal probe and quenching the signal of the first signal probe when hybridized in quenching proximity thereto, said first quencher probe having a  $T_m$  below that of the first signal probe; (iii) at least a second signal probe which is capable of hybridizing to at least a portion of a second target sequence and producing a second detectable signal when hybridized thereto; and (iv) an optional second quencher probe which is capable of hybridizing in quenching proximity to the second signal probe and quenching the signal of the second signal probe when hybridized in quenching proximity thereto, said optional second quencher probe having a  $T_m$  below that of the second signal probe;

monitoring the detectable signals of the signal probes as a function of temperature; and

determining therefrom the presence or absence of one or more target sequences in said polynucleotide sample.

2. The method of Claim 1 in which the first and second detectable signals are fluorescent signals.

3. The method of Claim 2 in which the first and second fluorescent signals are spectrally resolvable.

4. The method of Claim 1 in which the  $T_m$  of the first signal probe is higher than the  $T_m$  of the second signal probe.

5. The method of Claim 1 in which the  $T_m$  of the first quencher probe is in the range of about 5 to 10°C lower than that of the first signal probe and the  $T_m$  of the optional second quencher probe is in the range of about 5 to 10°C lower than that of the second signal probe.

6. The method of Claim 2 in which the first and second fluorescent signals are not spectrally resolvable, and the second signal probe has a lower  $T_m$  than the first quencher probe.

7. The method of Claim 6 in which the  $T_m$  of the first quencher probe is in the range of about 5 to 10°C lower than that of the first signal probe and the  $T_m$  of the optional second quencher probe is in the range of about 5 to 10°C lower than that of the second signal probe.

8. The method of Claim 6 in which the  $T_m$  of the second signal probe is in the range of about 7 to 15°C lower than that of the first signal probe.

9. The method of Claim 6 in which the first and second fluorescent signals are the same.

10. The method of Claim 1 in which the optional second quencher probe is present.

11. The method of Claim 1 in which the first and second signal probes are self-indicating signal probes.

12. The method of Claim 11 in which the self-indicating probes are hairpin probes.

13. The method of Claim 12 in which the first signal, first quencher, second signal and optional second quencher probes are resistant to degradation by nucleases.

**14.** The method of Claim 12 in which the first signal, first quencher, second signal and optional second quencher probes are each, independently of one another, selected from the group consisting of a DNA nucleobase oligomer, an RNA nucleobase oligomer and a PNA nucleobase oligomer.

**15.** The method of Claim 14 in which the first signal, first quencher, second signal and optional second quencher probes are all DNA, RNA or PNA nucleobase oligomers.

**16.** The method of Claim 11 in which the self-indicating probes are linear self-indicating probes.

**17.** The method of Claim 16 in which the first signal, first quencher, second signal and optional second quencher probes are resistant to degradation by nucleases.

**18.** The method of Claim 16 in which the first signal, first quencher, second signal and optional second quencher probes are each, independently of one another, selected from the group consisting of DNA, RNA and PNA nucleobase oligomers.

**19.** The method of Claim 18 in which the first signal, first quencher, second signal and optional second quencher probes are all DNA, RNA or PNA nucleobase oligomers.

**20.** The method of Claim 18 in which the first signal, first quencher, second signal and optional second quencher probes are all PNA nucleobase oligomers.

**21.** The method of Claim 11 in which each self-indicating probe includes a label which is capable of distinguishing hybridized from unhybridized signal probe.

**22.** The method of Claim 21 in which the label is a fluorescent intercalating dye.

23. The method of Claim 22 in which the fluorescent intercalating dye is selected from the group consisting of acridine orange, ethidium bromide, propidium iodide, hexium iodide, ethidium bromide homodimer, 3,3'-diethylthiadicarbocyanine iodide, SYBR<sup>®</sup> Green I and SYBR<sup>®</sup> Green II, 7-aminoactinomycin D, and actinomycin D.
24. The method of Claim 21 in which the label is a fluorescent minor-groove-binding dye.
25. The method of Claim 24 in which the fluorescent minor-groove-binding dye is selected from the group consisting of bisbenzimidazole dyes such as Hoechst 33258, Hoechst 33342, and Hoechst 34580 and indole dyes such as DAPI (4',6-diamino-2-phenylindole).
26. The method of Claim 1 in which the detectable signals are monitored as a function of decreasing temperature from a temperature above the  $T_m$  of the first signal probe to a temperature below the  $T_m$  of the optional second quencher probe.
27. The method of Claim 26 in which the detectable signals are monitored at temperatures approximately equal to the  $T_m$ s of the signal and quencher probes.
28. The method of Claim 26 in which the detectable signals are monitored at temperatures approximately halfway between the  $T_m$ s of the signal and quencher probes.
29. The method of Claim 26 in which the temperature is decreased at a rate in the range of about 0.01 °C/minute to about 5 °C/minute.
30. The method of Claim 26 in which the detectable signals are monitored continuously at a rate in the range of about every 100 to 10,000 msec as a function of temperature.
31. The method of Claim 1 in which the detectable signals are monitored as a function of increasing temperature from a temperature below the  $T_m$  of the

optional second quencher probe to a temperature above the  $T_m$  of the first signal probe.

32. The method of Claim 31 in which the detectable signals are monitored at temperatures approximately equal to the  $T_m$ s of the signal and quencher probes.

33. The method of Claim 31 in which the detectable signals are monitored at temperatures halfway between the  $T_m$ s of the signal and quencher probes.

34. The method of Claim 31 in which the temperature is increased at a rate in the range of about 0.01 °C/minute to about 5 °C/minute.

35. The method of Claim 31 in which the detectable signals are monitored continuously at a rate in the range of about every 100 to 10,000 msec as a function of temperature.

36. The method of Claim 1 in which the detectable signals are monitored as a function of temperature by determining the  $T_m$ s of the first and second signal probes.

37. The method of Claim 1 in which the polynucleotide sample is selected from the group consisting of genomic DNA, cDNA, RNA, mRNA, rRNA and an amplification product.

38. The method of Claim 37 in which the polynucleotide sample is single-stranded.

39. The method of Claim 1 in which the polynucleotide sample comprises two or more different polynucleotides.

40. The method of Claim 1 in which the target sequences are present on two or more polynucleotides.

41. The method of Claim 1 in which the target sequence is present on the same polynucleotide strand.

42. The method of Claim 1 in which the target sequence is present on two different polynucleotide strands.

43. A method of analyzing a polynucleotide sample for one or more target sequences, comprising the steps of:

contacting a polynucleotide sample with: (1) a first set of  $m$  signal-quencher probe pairs, each of which comprises (i) a signal probe capable of hybridizing to a portion of a target sequence and producing a first detectable signal when hybridized thereto and (ii) a corresponding quencher probe capable of hybridizing in quenching proximity to the signal probe and quenching its detectable signal when hybridized in quenching proximity thereto, wherein the first signal probe has the highest  $T_m$  and the  $T_m$  of each quencher probe is lower than the  $T_m$  of its corresponding signal probe and the  $T_m$  of each signal probe is lower than the  $T_m$  of the quencher probe of the preceding signal-quencher probe pair, and further wherein the quencher probe of the signal-quencher probe pair of the first set having the lowest  $T_m$  is optional; and (2) a second set of  $n$  signal-quencher probe pairs, each of which comprises (i) a signal probe capable of hybridizing to a portion of a target sequence and producing a second detectable signal distinguishable from the first detectable signal when hybridized thereto and (ii) a corresponding quencher probe capable of hybridizing in quenching proximity to the signal probe and quenching its detectable signal when hybridized in quenching proximity thereto, wherein the  $T_m$  of each quencher probe is lower than the  $T_m$  of its corresponding signal probe and the  $T_m$  of each signal probe is lower than the  $T_m$  of the quencher probe of the preceding signal-quencher probe pair, and further wherein the quencher probe of the signal-quencher probe pair of the second set having the lowest  $T_m$  is optional;

monitoring the first and second detectable signals as a function of temperature; and

determining the presence or absence of one or more target sequences in said polynucleotide sample.

**44.** A method of genotyping an organism, comprising the steps of:

contacting a polynucleotide sample from the organism, or an amplification product thereof, with a first plurality of signal-quencher probe pairs, each of which is capable of hybridizing, in quenching proximity, to a different genotype-specific sequence and producing a resolvable, temperature-dependent on-off hybridization profile;

obtaining temperature-dependent on-off hybridization profiles for the signal-quencher probe pairs; and

determining therefrom the genotype of the organism.

**45.** A method of genotyping a virus, comprising the steps of:

contacting a polynucleotide sample from a virus, or an amplification product thereof, with a first plurality of signal-quencher probe pairs, each of which is capable of hybridizing, in quenching proximity, to a different virus genotype-specific sequence and producing a resolvable, temperature-dependent on-off hybridization profile;

obtaining temperature-dependent on-off hybridization profiles for the signal-quencher probe pairs; and

determining therefrom the genotype of the virus.

**46.** A method of analyzing a sample for the presence of a polynucleotide sequence of interest, comprising the steps of:

contacting a polynucleotide from the sample, or an amplification product thereof, with a first plurality of signal-quencher probe pairs, wherein each said signal-quencher probe pair is capable of hybridizing, in quenching proximity, to a different known target sequence and producing a resolvable, temperature-dependent, on-off hybridization profile;

obtaining temperature-dependent, on-off hybridization profiles for the signal-quencher probe pairs; and

determining the presence or absence of one or more different target sequences.

47. A multiplex method of genotyping a polynucleotide of an organism, comprising the steps of:

amplifying the polynucleotide in the presence of amplification primers suitable for producing a plurality of genotype-specific amplicons and a plurality of signal-quencher probe pairs, wherein each said signal-quencher probe pair is capable of hybridizing, in quenching proximity, to a different genotype-specific amplicon and producing a resolvable, temperature-dependent, on-off hybridization profile;

obtaining temperature-dependent, on-off hybridization profiles for the signal-quencher probe pairs; and

determining therefrom the genotype of the organism.

48. A multiplex method of diagnosing a patient for a malady of interest, comprising the steps of:

incubating a polynucleotide sample derived from the patient in the presence of a plurality of signal-quencher probe pairs, wherein each said signal-quencher probe pair is capable of hybridizing, in quenching proximity, to a different target sequence indicative of a particular malady of interest and producing a resolvable, temperature-dependent, on-off hybridization profile when hybridized thereto;

obtaining temperature-dependent, on-off hybridization profiles for the signal-quencher probe pairs; and

determining therefrom whether the patient has the malady of interest.



49. A multiplex method of diagnosing a patient for a malady of interest, comprising the steps of:

amplifying a polynucleotide sample derived from the patient in the presence of amplification primers suitable for producing a plurality of different amplicons, each of which correlates to a different malady of interest, and a plurality of signal-quencher probe pairs, wherein each said signal-quencher probe pair is capable of hybridizing, in quenching proximity, to a different amplicon and producing a resolvable, temperature-dependent, on-off hybridization profile;

obtaining temperature-dependent, on-off hybridization profiles for the signal-quencher probe pairs; and determining therefrom whether the patient has the malady of interest.

50. A kit for multiplex polynucleotide analysis, comprising a plurality of signal-quencher probe pairs, each of which pairs is capable of hybridizing, in quenching proximity, to a different target sequence and producing a resolvable, temperature-dependent on-off hybridization profile in the presence of its respective target sequence.

51. A kit for determining the presence or absence of mutations or polymorphisms at multiple loci of one or more polynucleotides, comprising a first signal probe which is capable of hybridizing to a polynucleotide at a region of a first target sequence and producing a first detectable fluorescent signal when hybridized thereto, a first quencher probe capable of hybridizing, in quenching proximity, to a different region of the same target sequence as the first signal probe and quenching the signal of the first signal probe when hybridized in quenching proximity thereto, said first quencher probe having a  $T_m$  below that of the first signal probe, a second signal probe which is capable of hybridizing to the same or different polynucleotide at a region of a second target sequence and producing a second detectable fluorescent signal when hybridized thereto, said second signal probe having a  $T_m$  below that of the first quencher probe, and an optional second quencher probe which is capable of

hybridizing, in quenching proximity, to the a different region of the same target sequence as the second signal probe and quenching the signal of the second signal probe when hybridized in quenching proximity thereto, said optional second quencher probe having a  $T_m$  below that of the second signal probe.